ISSN 1346–7565 Acta Phytotax. Geobot. 58 (2/3): 112–118 (2007)

SHORT COMMUNICATION

Karyological Observations in *Musa beccarii* var. *hottana* (Musaceae)

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The center of diversity of the genus *Musa* (Musaceae) is in Southeast Asia, a region not studied in detail and where new species and varieties continue to be reported. Recently, a new variety, *Musa beccarii* var. *hottana* was described. To improve its characterization, the chromosome number and genome size of varieties *hottana* and *beccarii* were analyzed. Variety *hottana* had a diploid number of $2n = 18$, the same as var. *beccarii*. This is an interesting finding, as other species of *Musa* section *Callimusa*, in which *M. beccarii* has traditionally been included, have $2n = 20$. Nuclear DNA amounts (2C) of var. *beccarii* and var. *hottana* were estimated to be 1.562 pg and 1.645 pg, respectively. These are the largest genome sizes reported so far in *Musa*. The difference in genome size between the two varieties (~5%) is highly significant and diagnostic, implying that DNA flow cytometry may be used to discriminate between them and to identify the endangered var. *hottana*. The analysis of the genome structure of *M. beccarii* may contribute to an understanding of the processes at the DNA and chromosomal levels that accompanied the evolution in the genus *Musa*.

Key words: *Callimusa*, Chromosome number, Flow cytometry, Genome size, *Musa*, *Musa beccarii* var. *hottana*, Nuclear DNA content, Wild banana.

The family Musaceae includes three genera, *Musa* L., *Ensete* Horan. and *Musella* (Franch.) C. Y. Wu (Horaninow 1862, Franchet 1889, Wu 1978). *Musa* includes both wild species and cultivated seed-sterile forms of enormous socio-economic importance, such as bananas and plantains. It has been estimated that *Musa* comprises about 70 species. Many regions within its center of diversity in Southeast Asia have not been explored systematically and new species continue to be discovered. Recently described species include *M. bauensis* Häkkinen & Meekiong (2004), *M. azizii* Häkkinen (2005) and *M. barioensis* Häkkinen (2006a).

Musa has traditionally been divided into four sections based on chromosome number: *Australimusa* with $2n = 20$, *Callimusa* with $2n = 20$, *Eumusa* with $2n = 22$ and *Rhodochlamys* with $2n = 22$ (Cheesman 1947). This classification, however, has often been questioned and DNA analyses have pointed to its shortcomings (Lanaud *et al.* 1992, Gawel *et al.* 1992, Jarret *et al.* 1992, Ude *et al.* 2002a, 2002b). Wong *et al.* (2002) therefore proposed to group the species of *Musa* into two sections, one including *Australimusa* and *Callimusa*, and the other including *Eumusa* and *Rhodochlamys*. Nwakanma *et al.* (2003) supported this view and suggested splitting the joint *Eumusa* section into two groups, *Eumusa*-1 and *Eumusa*-2.

Another problem in the taxonomy of *Musa* is in the placement of species that differ from other species in chromosome number. For example, *M. ingens* N. W. Simmonds with $2n = 14$ was treated as *incertae sedis* by Simmonds (1962). Argent (1976), however, created the new section *Ingentimusa* comprising only *M. ingens*, but this proposal has not been commonly accepted. *Musa beccarii* N. W. Simmonds is another "difficult" species as its chromosome number ($2n = 18$) differs from all other species of *Musa*. Interestingly, $2n = 18$ is characteristic for species of the related genera *Ensete* and *Musella*. *Musa beccarii* was described by N. W. Simmonds from Trinidad after observing plants grown from seeds that were imported from Borneo (Simmonds 1956). While *M. beccarii* was originally treated as *incertae sedis* by Simmonds (1962), more recent numerical taxonomic analysis (Simmonds & Weatherup 1990) and examination of its morphological characteristics (Häkkinen 2004a) have lead to placing *M. beccarii* within section *Callimusa*.

More recently, Häkkinen re-described *M. beccarii* var. *beccarii* during extensive field studies in Borneo (Häkkinen *et al.* 2005). During that expedition, as a consequence of massive land cleaning for oil palm plantations, only a few populations of *M. beccarii* were found. An import outcome of the field study was a discovery of a new variety, *M. beccarii* var. *hottana* Häkkinen (Häkkinen *et al.* 2005). Whereas var. *beccarii* grows in the open, var. *hottana* grows only under the canopy. Moreover, var. *hottana* appears to be extremely rare and until now has been found only in one location.

Preservation of rare species should be supported based on details of their characterization. In addition to the morphological distinction of the two varieties of *M. beccarii* (Häkkinen *et al.* 2005), Häkkinen *et al.* (2007) analyzed them using Inter-Retrotransposon Amplified Polymorphism (IRAP). The high degree of IRAP polymorphism detected in their study indicates that varieties *beccarii* and *hottana* should be considered distinct taxa. Additional IRAP bands found in var. *beccarii* indicate that var. *hottana* may be evolutionarily more ancient than var. *beccarii*.

While the use of molecular markers in systematics and taxonomy continues to increase, chromosome number, morphology, and nuclear genome size are the basic characteristics of a species. Flow cytometry allows rapid and precise estimation of genome size (Doležel & Bartoš 2005) and has successfully been used in evolutionary and population biology, including for the detection and delineation of species and to infer phylogenetic relationships (Kron *et al.* 2007). The chromosome number of *M. beccarii* var. *beccarii* was determined by Shepherd (1959) to be $2n = 18$. Until now, the chromosome number of *M. beccarii* var. *hottana* was unknown. This study for the first time establishes the chromosome number and nuclear genome size of var. *hottana* using flow cytometry.

Materials and Methods

Plant material

Living plants of *M. beccarii* var. *hottana* were collected from the Kinanbatangan River delta area, Sabah, Malaysia, (Voucher specimen: Malaysia, Sabah, Kinanbatangan River delta area, 05° 30.749′N., 118°17.361′E, 23 m elevation, 20 May 2004, *M. Häkkinen & J. Gisil 12* [BORH]. Suckers were transferred to the Institute for Tropical Biology and Conservation, Universiti Malaysia, Sabah, and to the University of Helsinki, Finland, for further growth. T To obtain seeds, the flowers of *M. beccarii* var. *hottana* (*2005-0826* [H]) were hand pollinated using pollen from other staminate plants of var. *hottana* in the University of Helsinki greenhouse. The seeds were collected about 3 months later and were germinated in garden compost. Young seedlings were potted and shipped to the Institute of Experimental Botany in Olomouc, Czech Republic. *Musa beccarii* var. *beccarii* (*ITC 1070*) was obtain-

ed from the INIBAP Transit Centre (ITC), Katholieke Universiteit Leuven, Belgium, as *in vitro* rooted plantlets. After transfer to soil, the plants were maintained in a greenhouse.

Chromosome preparations

Metaphase spreads of root tips were prepared as described by Doleželová *et al.* (1998). Actively growing root tips were pre-treated in 0.05% 8-hydroxyquinoline for 3 hrs and fixed in 3:1 ethanol: acetic acid. Fixed roots were washed in a solution of 75 mM KCl and 7.5 mM EDTA (pH 4). Meristem tips were digested in a mixture of 2% pectinase and 2% cellulase for 90 min at 30°C. Protoplast suspension was filtered through a 150 μm nylon mesh and pelleted. The pellet was resuspended in 75 mM KCl and 7.5 mM EDTA (pH 4) and incubated for 5 min at room temperature. After pelleting, the protoplasts were washed three times with 70% ethanol, and 5 μL of suspension were dropped onto a slide. Shortly before drying out, 5 μL of 3:1 fixative were added to the drop to induce protoplast bursting. Finally, the slide was rinsed in 100% ethanol and air-dried. The chromosomes were stained in 3% Giemsa solution for 25 min at room temperature. After washing in distilled water, the slides were mounted in Euparal (Carl Roth, Karlsruhe, Germany) and observed with an Olympus AX70 microscope using a 100x/1.35 oil immersion objective. Images were recorded using a CCD camera interfaced to a PC running the MicroImage software (Olympus, Tokyo, Japan). For each variety, three plants were analyzed and in each plant 10 - 15 metaphase spreads were evaluated for chromosome number.

Determination of nuclear genome size

Approximately 50 mg of midrib was cut from a young *Musa* leaf and transferred to a glass Petri dish. About 10 mg of a young leaf of soybean (*Glycine max* L. 'Polanka') with 2C = 2.5 pg DNA (Doležel *et al.* 1994) was added to serve as an internal reference standard. Both tissues were chopped simultaneously in 1 mL Otto I solution (0.1 M citric acid, 0.5 % v/v Tween 20; Otto 1990). The crude suspension of isolated nuclei was filtered through a 50 μm nylon mesh. Nuclei were then pelleted (300 g, 5 min), resuspended in 200 μL Otto I and incubated for 1hour at room temperature. Finally, 600 μL Otto II solution (0.4 M Na_2HPO_4; Otto 1990), supplemented with 15 mM β-mercaptoethanol, 50 μg/mL RNase and 50 μg/mL propidium iodide (PI)

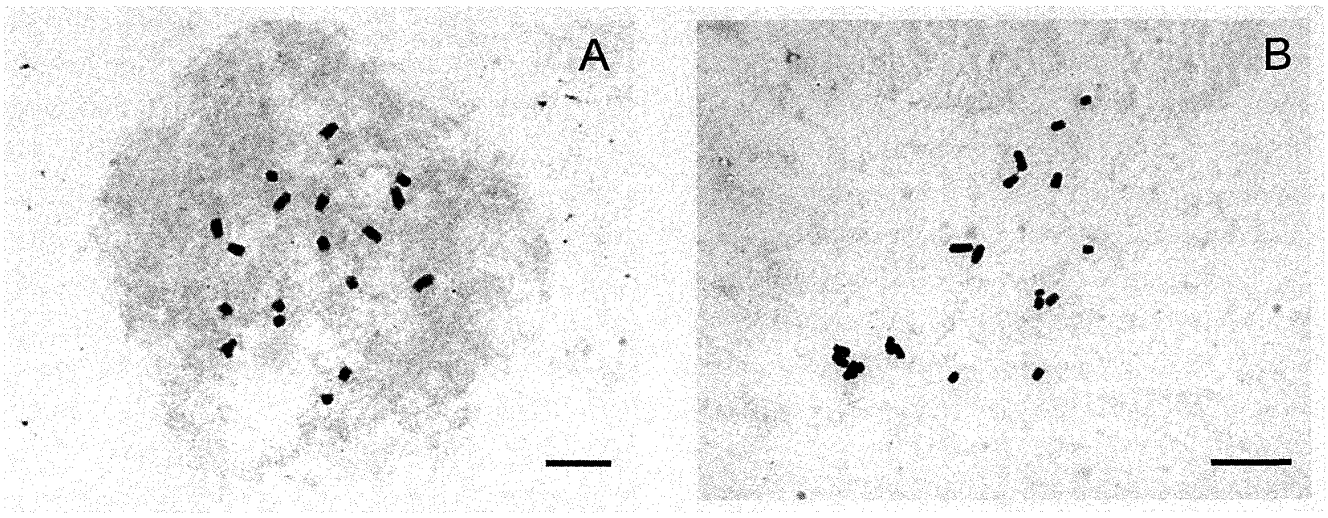

FIG. 1. Mitotic metaphase spreads of *Musa beccarii*. A: *M. beccarii* var. *beccarii* (2*n* = 18). B: M. *beccarii* var. *hottana* (2*n* = 18). Bar = 10 μm.

was added. Samples were analyzed using Partec PAS flow cytometer (Partec, Münster, Germany) equipped with 488-nm argon ion laser. The gain of the instrument was adjusted so that the G_0/G_1 peak of *Glycine* was approximately on channel 200, and at least 5,000 nuclei were analyzed in each sample at a rate of 20 - 35 nuclei/sec. Three plants were measured per variety. Analysis of each plant was repeated three times on three different days. Nuclear DNA content was calculated from individual measurements following the formula:

2C Nuclear DNA content = $2.5 \times G_0/G_1$ peak mean of *Musa* / G_0/G_1 peak mean of *Glycine* [pg DNA, channel number]

Mean nuclear DNA content was then calculated for each plant. Genome size, which represents one copy of nuclear genetic information (equal to 1C), was determined considering 1 pg DNA to be equal to 0.978×10^9 bp (Doležel *et al.* 2003). Data analysis was done using nested ANOVA with the NCSS 97 statistical software (Statistical Solutions Ltd, Cork, Ireland).

Result and Discussions

During an extensive field study of wild bananas in 2001, 2002 and 2004 in Sarawak, Brunei and Sabah, the first author studied species of section *Callimusa*. As a result, varieties of *M. beccarii i.e. beccarii* and *hottana* were determined to belong to section *Callimusa*, and were grouped with the following species and varieties with $2n = 20$: 1) *Musa azizii*, 2) *M. bauensis*, 3) *M. barioensis*, 4) *M. campestris* [*i.e.* var. *campestris*, var. *lawasensis*, var. *limbangensis*, var. *miriensis*, var. *sabahensis*, var. *sarawakensis*], 5) *M. hirta*, 6) *M. lawitiensis* [*i.e.* var. *lawitiensis*, var. *kapitiensis*, var. *sarawakensis* and var. *suratii*], 7) *M. monticola*, 8) *M. muluensis*, 9) *M. tuberculata*, 10) *M. voonii*. The grouping was based on their similarity in morphology characteristics and seed forms, and ignored the incongruous number of chromosomes. The other clearly distinct group of varieties in section *Callimusa* with $2n = 20$, based on their morphology and seed shape,

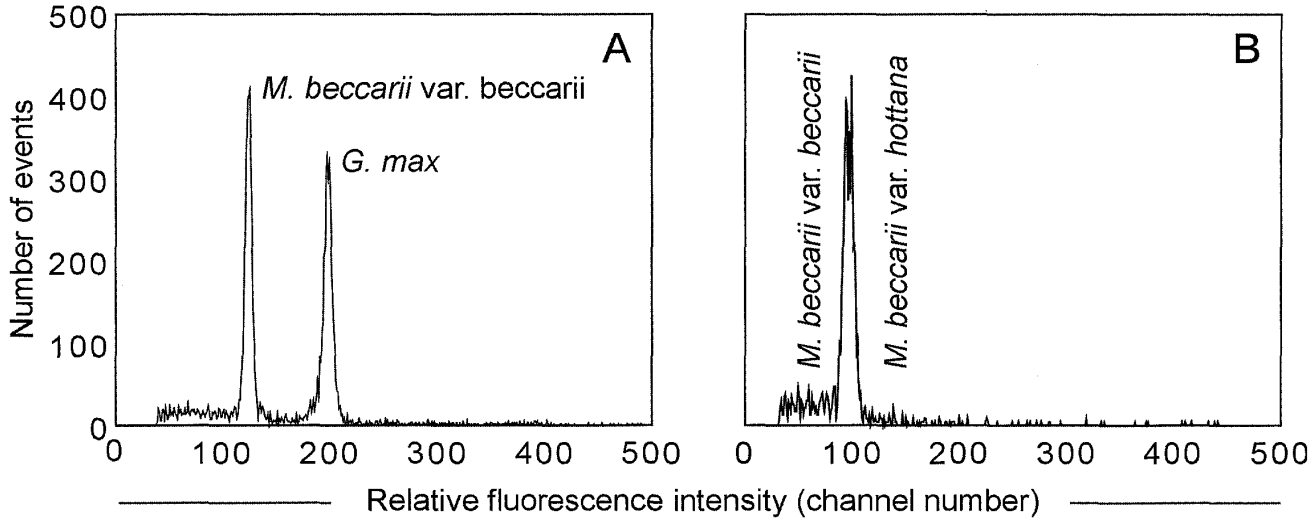

FIG. 2. Flow cytometric estimation of nuclear DNA content in *Musa beccarii*. A: Histogram of relative DNA content obtained after simultaneous analysis of nuclei isolated from *M. beccarii* var. *beccarii* and *Glycine max* 'Polanka'. Peaks representing G_0/G_1 nuclei of *M. beccarii* (mean channel number 125.86) and *Glycine max* 'Polanka' (mean channel number 200.58), which served as internal reference standard, are clearly visible. B: Simultaneous analysis of nuclei isolated from *M. beccarii* var. *hottana* and *M. beccarii* var. *beccarii*. The partial overlap of G_0/G_1 peaks resulted in a bimodal peak with peak modes at channels 97 and 102 for var. *beccarii* and var. *hottana*, respectively.

are the following species and varieties in Borneo: *M. borneensis* [*i.e.* var. *borneensis*, var. *alutacea*, var. *flavida*, var. *lutea*, var. *phoenicea* and var. *sarawakensis*] (Häkkinen 2003, 2004a, 2004b, 2005, 2006a, 2006b, Häkkinen & Meekiong 2004, 2005, Häkkinen *et al.* 2005).

In this study, the chromosome number of *M. beccarii* var. *hottana* was determined for the first time as $2n = 18$ (Fig. 1A). The same chromosome number ($2n = 18$) was observed also in *M. beccarii* var. *beccarii* (Fig. 1B), which is in line with previous observations (Simmonds 1956, Bartoš *et al.* 2005). The chromosome size at mitotic metaphase after 8-hydroxyquinoline treatment ranged from 1.5 to 3 μm in both varieties.

Flow cytometric analysis of propidium iodide-stained nuclei resulted in histograms of relative nuclear DNA content with two dominant peaks corresponding to G_0/G_1 nuclei of *Musa* and *Glycine* respectively (Fig. 2A). The coefficients of variation (CVs) of the DNA peaks ranged from 2.2 to 3.6 % both for *Musa* and *Glycine* throughout the whole experiment. Nuclear DNA content was determined based on the ratio of G_0/G_1 peak positions: *M. beccarii* var. *beccarii* had 2C = 1.562 ± 0.014 pg DNA (mean ± SD, $N = 3$), while var. *hottana* had 2C = 1.645 ± 0.011 pg DNA (mean ± SD, $N = 3$). This approximately corresponds to 1C genome sizes of 764 Mbp for var. *beccarii* and 804 Mbp for var. *hottana*. Thus the nuclear genome of var. *hottana* is about 5% larger than that of var. *beccarii*. Nested ANOVA indicated that this difference was significant ($P < 0.001$). No statistically significant variation in DNA content between plants within a variety and between replicate measurements of the same plant was found.

To confirm this observation, nuclei from both varieties were isolated, stained and analyzed simultaneously. The resulting distribution of DNA content comprised a bimodal peak resulting from a partial overlap of G_0/G_1 peaks of both varieties (Fig. 2B). The CV of the bimodal peak was 5.45 ± 0.29% (mean ± SD, $N = 4$), *i. e.* about twice the CVs of the G_0/G_1 peaks of individual varieties, and reflected partial peak overlap. This observation confirmed the difference in genome size between var. *beccarii* and var. *hottana*. The observed differentiation of the genome of var. *hottana* from that of var. *beccarii*, presumably due to an increase in copy number of some DNA sequences, may have been facilitated by its geographic isolation and may represent an early step towards speciation. This process might be stimulated by the relatively small population size.

Our observations imply that DNA flow cytometry may be used to discriminate between the varieties of *Musa beccarii*. In a similar study, Doležel *et al.* (1994) revealed a 10% difference between the genome size of *M. acuminata* Colla and *M. balbisiana* Colla (Colla 1820). That difference was found to be diagnostic and has been used not only to discriminate between both genomes but also to predict the genomic constitution of their hybrids (Lysák *et al.* 1999). As there is currently no simple molecular assay to discriminate varieties *beccarii* and *hottana*, flow cytometry may be instrumental in identifying the endangered variety *hottana*.

The present estimates of 2C DNA amounts in *M. beccarii* are in line with those of Bartoš *et al.* (2005), who analyzed *M. beccarii* var. *beccarii*. Until now the largest nuclear genome sizes observed in *Musa* were 1.377 pg DNA/2C in species in section *Eumusa*, 1.299 pg DNA/2C in section *Rhodochlamys*, 1.547 pg DNA/2C in section *Australimusa* and 1.561 pg DNA/2C in section *Callimusa* (Bartoš *et al.* 2005). Thus, *M. beccarii* possesses the largest nuclear genome so far known in *Musa*, with var. *hottana* having the single largest genome. This is interesting as *M. beccarii* has the smallest number of chromosomes *Musa* except for *M. ingens* with $2n = 14$ (Simmonds 1960). Interestingly too, *M. beccarii* has the same chromosome number as the species in the related genera *Ensete* and *Musella*. Simmonds (1962) speculated that the lower chromosome number of *M. beccarii* and the species of *Ensete*, togeth-

er with their restricted geographic distribution, reflect their ancient origin, and he considered them relics in the evolution of Musaceae. If so, the analysis of their genomes at the DNA and chromosomal levels could provide important clues to the evolution of the interesting taxonomic groups and clarify their classification.

Currently available data do not permit reaching a conclusion on karyotype evolution in *Musa*. If *Ensete* and *Musella* are considered relicts in the evolution of *Musa*, then evolution from a common ancestor was accompanied by an increase in the number of chromosomes (chromosome fission). As the genome size in *Ensete* and in species of *Eumusa* is similar (Bartoš *et al.* 2003), the larger genome of *M. beccarii* may have resulted from duplication /amplification of DNA after the separation from the main lineage. Alternatively, speciation within *Ensete* and *Eumusa* was accompanied by elimination of some DNA. Future work should involve analysis of repeated parts of the genomes as well as comparative karyotype analysis using fluorescence *in situ* hybridization (FISH) to study genomic distribution of selected repetitive DNA sequences and structural changes that differentiate the karyotype of *M. beccarii* from related species.

The first author greatly appreciates the help and support of the staff of the Institute for Tropical Biology during the study in Sabah. We thank Ir. Ines van den Houwe, *Musa* curator of the INIBAP Transit Centre at the Katholieke Universiteit, Leuven, Belgium, for supplying *Musa beccarii* var. *beccarii* ITC 1070.

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Received July 5, 2007; accepted September 29, 2007